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Epstein-Barr Virus-Specific Antibodies in Epstein-Barr Virus-Positive and -Negative Gastric Carcinoma Cases in Japan

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We examined Epstein-Barr virus (EBV)-specific antibodies in serum samples from 64 and 59 patients with EBV-positive and -negative gastric carcinomas, respectively, and 73 healthy controls using immunofluorescence assays. EBV capsid antigen (VCA) IgG and EBV-determined nuclear antigen (EBNA) IgG were detected in all 196 subjects. The geometric mean titer (GMT) of VCA-IgG, but not EBNA-IgG, was higher in EBV-positive carcinoma cases than in EBV-negative carcinoma cases ($P < 0.001$). The seroprevalence rates of VCA-IgA and EBV early antigen (EA) IgG were higher in EBV-positive carcinoma cases than in EBV-negative carcinoma cases. Odds ratios (ORs) comparing seroprevalence rates between EBV-positive and -negative carcinoma cases were 3.4 (95% confidence interval [CI] = 1.3–8.8) and 6.6 (95% CI = 2.7–16.3) for VCA-IgA and EA-IgG, respectively. These results suggest that EBV reactivation occurs in vivo, since more than 90% of Japanese are infected with EBV in early childhood. The GMT of VCA-IgG in EBV-negative carcinoma cases was higher than that of healthy controls ($P = 0.028$). The seroprevalence rates of EA-IgG were greater in EBV-negative carcinoma cases than in healthy controls (OR = 4.9, 95% CI = 1.2–19.7). VCA-IgA was the only antibody that showed a significantly high seroprevalence and GMT in EBV-positive carcinoma cases, but not in EBV-negative carcinoma cases. Thus, VCA-IgA can be a marker of immune response to EBV in EBV-positive carcinoma cases. Our findings support the hypothesis that if EBV is involved in the development of EBV-positive gastric carcinoma, the EBV reactivation occurs in vivo. ***J. Med. Virol. 60:411–416, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: Epstein-Barr virus-determined nuclear antigen IgG; Epstein-Barr virus capsid antigen IgG; Epstein-Barr virus capsid antigen IgA; Epstein-Barr virus early antigen IgG; epidemiology

INTRODUCTION

Epstein-Barr virus (EBV) involvement has been noted in 6.7% of gastric carcinomas of a Japanese series [Tokunaga et al., 1993a] and in 16% of a North American series [Shibata and Weiss, 1992] using in situ hybridization (ISH) to detect EBV-encoded small RNA (EBER)-1 in the nucleus of cells latently infected with EBV [Chang et al., 1992]. EBER-1-positive gastric carcinoma (EBV-positive gastric carcinoma) is known for the uniform expression of EBV-determined nuclear antigen (EBNA)-1 and EBER in all carcinoma cells, the episomal monoclonality of the EBV genome, and elevated antibodies against EBV-related antigens [Tokunaga et al., 1993b; Imai et al., 1994]. In addition, the unique "lace pattern" morphologic features have been observed in some early-stage EBV-positive gastric carcinomas and are characterized by lymphocytic infiltration in and around the carcinoma nests in the mucosa [Uemura et al., 1994]. These findings suggest that EBV

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plays an important role in the origin of EBV-positive gastric carcinoma.

EBV infection is so common in Japan that most Japanese are infected with EBV in childhood [Mizuno, 1990]. Although EBV is maintained as a lifelong infection in vivo, it is usually latent, and the majority of viral carriers are asymptomatic during their lives. And even though serum antibody titers against EBV capsid antigen (VCA) and EBV early antigen (EA) are stable or negative in asymptomatic viral carriers, the levels of these antibodies are boosted in immunocompromised hosts, such as patients who have undergone organ transplantation and are under immunosuppressive treatment [Rickinson and Kieff, 1996].

On the other hand, it is well known that EBV infection of B lymphocytes in vitro results in immortalized cell lines. EBV is also suspected of causing malignant neoplasms, including Burkitt's lymphoma in Africa [Zur Hausen et al., 1970], nasopharyngeal carcinoma (NPC) in southern China [Deng et al., 1995], Hodgkin's disease [Weiss et al., 1989], and B-cell lymphoma in immunocompromised individuals [McMahon et al., 1991]. Patients with these malignant diseases show unusual elevations of EBV-specific antibodies in serum samples [Zur Hausen et al., 1970; de-The et al., 1978; Mueller et al., 1989], including antibodies against VCA and EA. Furthermore, some studies have suggested that EBV might be reactivated in vivo before the development of these diseases [Zur Hausen et al., 1970; Mueller et al., 1989]. To investigate the relationship between immune response against EBV and the risk of gastric carcinoma in Japanese patients, we compared the seroprevalence and geometric mean titers (GMTs) of EBV-specific antibodies among EBV-positive and -negative gastric carcinoma patients and healthy controls.

MATERIALS AND METHODS

Subjects

Ninety EBV-positive and 107 EBV-negative carcinoma cases were chosen from the files of 3,152 gastric carcinomas diagnosed at Kagoshima City Hospital (Kagoshima, Japan) during the period 1992 to 1995. After reconfirmation of cancer diagnosis with hematoxylin-eosin staining, EBER ISH was carried out with a digoxigenin-labeled EBER-1 oligonucleotide probe [Chang et al., 1992]. A case was considered to be EBV positive based on a positive signal under microscopy. We first examined the presence of EBER-1 in the biopsy specimen by EBER ISH and then confirmed the results in the permanent pathologic specimen. One hundred and nine healthy controls were recruited from persons attending a health checkup at Kagoshima Medical Laboratory Center in 1994. After the exclusion of patients who had no pathologic and demographic information for our analysis, we were left with 64 EBV-positive patients with carcinoma, 59 EBV-negative patients with carcinoma, and 73 healthy controls.

Collection of Blood Samples

Most of the blood samples from the EBV-positive patients with carcinoma were collected within a 2-week period before gastrectomy, while the samples from the EBV-negative carcinoma patients were taken after gastrectomy, but within 1 week in most cases and no longer than 2 weeks in the rest. Blood samples of the healthy controls were collected when the subjects came to Kagoshima Medical Laboratory Center for a health checkup between June and August in 1994. All serum samples were separated within 24 hours and stored at –30°C until antibody assays were conducted.

Immunofluorescence

Indirect immunofluorescence assays were undertaken to examine antibodies against VCA (IgG, IgM, and IgA) and EA (IgG and IgA) [Henle et al., 1974]. An anticomplement immunofluorescence assay was performed to detect EBNA-IgG. Briefly, after diluting the serum with phosphate-buffered saline, antibodies against VCA and EBNA were detected in P3HR-1 and Raji cells, respectively [Henle et al., 1974; Yamamoto and Hinuma, 1976]. These cells were provided by Dr. Shousuke Imai [Imai et al., 1994]. To detect antibodies against EA, we used Raji cells superinfected with EBV derived from P3HR-1 [Yamamoto and Hinuma, 1976]. If the EBV-specific antibody was detected in serum diluted fivefold, the case was regarded as seropositive. Two different persons examined antibody titers and reported their judgments independently. When the two researchers disagreed, the lower antibody titer was always used in the analysis.

Pathologic Classification

Paraffin block samples of gastric specimens from the EBV-positive and -negative gastric carcinoma cases were used for the pathologic examinations. The tumor site and histopathologic type were classified according to the scheme of the Japanese Gastric Cancer Society [Japanese Research Society for Gastric Cancer, 1993]. The site of a tumor was classified as the cardia, middle portion, or antrum, according to its predominant location. The gastric remnant tumors were excluded because of their unique pathologic features [Yamamoto et al., 1994].

Follow-up Study

In 24 EBV-positive carcinoma cases, it was possible to follow the EBV-specific antibody titers of the patients after gastrectomy. However, only seven cases could be followed for more than 6 months. Blood samples were taken from each patient at least three times during the follow-up period.

Statistical Analysis

GMTs were calculated in the analysis of the antibody titers because the distribution of the antibody titers was positively skewed, with a long upper tail. Logistic analysis was conducted to compare the seroprevalence

TABLE I. Demographic Characteristics of the Study Subjects*

Characteristics	EBV-positive patients with carcinoma[a] n	(%)	EBV-negative patients with carcinoma[b] n	(%)	Healthy controls n	(%)
Sex						
Male	51	(79.7)	31	(52.5)	22	(30.1)
Female	13	(20.3)	28	(47.5)	51	(69.9)
Age (years)						
<40	3	(4.7)	3	(5.1)	4	(5.5)
40–49	5	(7.8)	5	(8.5)	37	(50.7)
50–59	14	(21.9)	9	(15.3)	22	(30.1)
60–69	23	(35.9)	21	(35.6)	8	(11.0)
70+	19	(29.7)	21	(35.6)	2	(2.7)

*EBV, Epstein-Barr virus.
[a]Patients with EBV-positive tumor.
[b]Patients with EBV-negative tumor.

rates and GMTs of EBV antibodies in the EBV-positive and -negative carcinoma cases. Log-transformed values were used in the logistic analysis of the antibody titers. Maximum likelihood estimates of odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) were obtained [Clayton and Hills, 1993]. All of the *P* values presented were two-sided.

RESULTS

Characteristics of the Study Subjects

We examined 64 and 59 serum samples obtained from EBV-positive and -negative patients with gastric carcinoma, respectively, and 73 serum samples from healthy controls. Table I shows the age and sex distributions of the study subjects. The distributions of age and sex were different among the three groups. The proportion of male patients was highest in the group with EBV-positive carcinoma and lowest among the healthy controls. The mean ages of the EBV-positive and -negative carcinoma patients and the healthy controls were 62.7 (standard deviation [SD] = 11.4), 63.8 (SD = 12.1), and 49.1 (SD = 9.3) years, respectively.

Seroprevalence of Epstein-Barr Virus-specific Antibody

As shown in Table II, all serum samples examined in the present study showed positive results for VCA-IgG and EBNA-IgG, regardless of the presence or absence of EBV genomes in gastric carcinomas. The results of the logistic analysis are shown in Table III. The seroprevalence rates of VCA-IgA and EA-IgG in the EBV-positive patients with carcinoma were significantly higher than those of the EBV-negative patients with carcinoma. The seroprevalence rate of EA-IgG was higher in the EBV-negative patients with carcinoma than in the healthy controls. Although we examined VCA-IgM in 10 EBV-positive patients with carcinoma, all of them tested negative.

Geometric Mean Titers of Epstein-Barr Virus-Specific Antibodies

Table II also shows the GMTs of antibodies. It was difficult to compare the GMT of EA-IgA in the EBV-positive and -negative carcinoma cases because of the small number of seropositive cases. We examined the logistic analysis for the log-transformed antibody titers of each antibody, adjusting for age and sex. The log titers of VCA-IgA ($P = 0.006$), EA-IgG ($P < 0.001$), and VCA-IgG ($P < 0.001$) were significantly higher in the EBV-positive carcinoma cases than in the EBV-negative carcinoma cases. With regard to EBNA-IgG, the antibody titers showed no difference between the EBV-positive and -negative carcinoma cases. The distribution patterns of VCA-IgG in the EBV-positive and -negative carcinoma cases are shown in Fig. 1. There were two peaks in the EBV-positive carcinoma cases, one at 640-fold dilution and the other at 2,560-fold dilution. The distribution in the EBV-positive carcinoma cases was shifted to the right-hand side, compared with that of the EBV-negative carcinoma cases. Figure 2 shows the distribution patterns of VCA-IgA. The peaks of the EBV-positive carcinoma cases were slightly shifted to the right-hand side when compared with the distribution in the EBV-negative carcinoma cases and in the healthy controls.

Pathologic Features

We also examined the relationship between pathologic features and the EBV-specific antibodies. In the EBV-positive carcinoma cases, VCA-IgA was much more frequently observed among patients with tumors in the cardia than at other tumor sites ($P = 0.005$). No such difference was found in the EBV-negative tumor cases. There was no association between the tumor site and any EBV antibody titers. None of the EBV-specific antibodies showed significant association with the depth of the tumor or any specific histologic type (data not shown).

Follow-Up Study

In seven of the 64 EBV-positive cases of carcinoma, we were able to examine the EBV-specific antibodies for at least 6 months after gastrectomy; the mean follow-up period was about 14 months. There was no change in antibody titer in six cases. One patient showed increases in VCA-IgG and EBNA-IgG titers 18 and 30 months after gastrectomy, respectively; a gradual increase in the VCA-IgG titer lasted until 30 months after operation. The VCA-IgA antibody titer was negative throughout the follow-up period, and this patient had no recurrence for at least 5 years after the operation.

DISCUSSION

In the present study, we observed significantly elevated antibody titers against VCA and EA, but not EBNA, in the EBV-positive gastric carcinoma cases compared with the EBV-negative carcinoma cases. Since more than 90% of Japanese are infected with EBV in early childhood [Mizuno, 1990], our results indicate that EBV reactivation occurs in vivo. This conclusion also is supported by the fact that VCA and EA are associated with the replicated cycle of EBV. EA-

TABLE II. EBV-specific Antibodies in Serum Samples of Patients with Gastric Carcinoma and Healthy Controls*

	EBV-positive patients with carcinoma (n = 64)		EBV-negative patients with carcinoma (n = 59)		Healthy controls (n = 73)	
Antibodies	Seropositive (%)	GMT[a]	Seropositive (%)	GMT	Seropositive (%)	GMT
VCA-IgG	100.0	1,733.5	100.0	678.7	100.0	433.6
VCA-IgA	34.4	6.1	13.6	<5	6.8	<5
EA-IgG	59.4	20.4	23.7	<5	6.8	<5
EA-IgA	1.6	<5	1.7	<5	0.0	<5
EBNA-IgG	100.0	56.6	100.0	49.4	100.0	63.1

*EBV, Epstein-Barr virus; VCA, EBV capsid antigen; EA, EBV early antigen; EBNA, EBV-determined nuclear antigen; GMT, geometric mean titer.
[a]The value of 2.5 is assigned for those with antibody titers under fivefold dilution.

TABLE III. Results of Logistic Analysis: Comparison of Seroprevalence Rates*

	EBV-positive patients relative to EBV-negative patients			EBV-negative patients relative to healthy controls		
	OR[a]	(95% CI)	*P* value	OR	(95% CI)	*P* value
VCA-IgA	3.4	(1.3–8.8)	0.009	2.6	(0.5–13)	0.235
EA-IgG	6.6	(2.7–16)	<0.001	4.9	(1.2–20)	0.02
EA-IgA	0.6	(0–9.9)	0.717	—	(–)	—

*EBV, Epstein-Barr virus; VCA, EBV capsid antigen; EA, EBV early antigen; OR, odds ratio; CI, confidence interval.
[a]Odds ratios and 95% confidence intervals were obtained from logistic analysis, adjusting for sex and age.

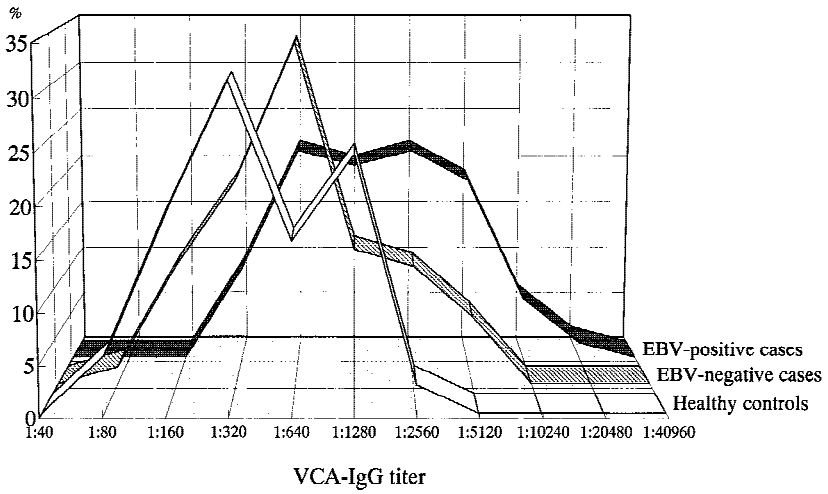

Fig. 1. Distribution patterns of Epstein-Barr virus (EBV) capsid antigen IgG in three groups. The EBV-positive carcinoma cases are indicated with dotted belt. The negative cases are indicated with oblique-lined belt. The healthy controls are indicated with gray belt.

and VCA-IgA are sensitive markers for NPC in its early stage [Henle and Henle, 1976] and are also predictors of disease prognosis [De Vathaire et al., 1988]. Since VCA-IgA was the only antibody showing significantly high seroprevalence and GMT in the EBV-positive carcinoma cases, but not in the EBV-negative carcinoma cases, VCA-IgA can be considered a marker of immune response to EBV in EBV-positive carcinoma cases. On the other hand, the seroprevalence rates and GMTs of VCA-IgG and we showed the details in results. EA-IgG except VCA-IgA were elevated in the EBV-negative patients with carcinoma when compared with the same parameters in the healthy controls. The cause of the elevated antibodies in the EBV-negative patients with carcinoma is unknown. Since the difference between these two groups was not notable, there was no denying that it was by chance.

Our results agree with the findings of the study carried out by Imai et al. [1994], which analyzed 14 patients each with EBV-positive and -negative gastric carcinoma and 24 healthy controls. They reported that the GMTs of VCA-IgG and EA-IgG were elevated among patients with EBV-positive tumors; the seroprevalence rates of EA-IgA and EA-IgG were much higher than those observed in the present study. Patients with other EBV-related malignant diseases, such as NPC and Hodgkin's disease, show elevated EBV-specific antibody titers before their diagnoses [Zur Hausen et al., 1970; de-The et al., 1978; Mueller et al., 1989]. Among patients with EBV-related diseases, including EBV-related gastric cancer, EBV reactivation in vivo could result from a defective cellular immune response to EBV. Levine et al. [1995] have reported that EBV-related gastric carcinoma patients

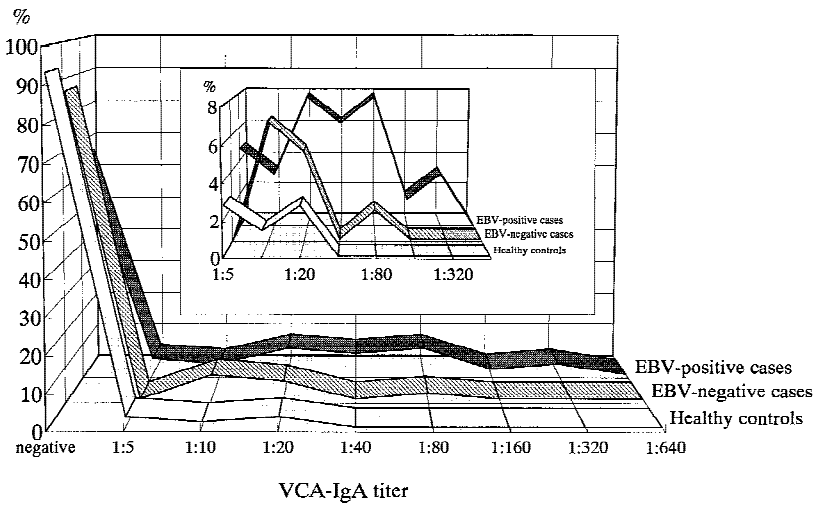

Fig. 2. Distribution patterns of Epstein-Barr virus capsid antigen IgA in three groups. The inset shows the distribution of only seropositive cases.

have significantly high antibody titers against VCA more than 5 years preceding their diagnoses. Serologic analysis for IgM to VCA or EA might clarify whether EBV reactivation is a current event within few months. In the present study, we examined VCA-IgM in10 EBV-positive patients with carcinoma, but none showed positive results. Because the reactivity pattern of VCA-IgM in vivo is still unknown, it is difficult to specify the period of viral reactivation.

In the follow-up study, most of the EBV-positive carcinoma cases did not show significant change in the VCA-IgG and EBNA-IgG antibody titers. Tamada et al. [1984] have observed decreased EBV-specific antibody titers in treated NPC patients after 6–20 months. Since EBV replication is found in NPC [Joab et al., 1991], but not in EBV-positive gastric carcinoma, the etiologic role of EBV might be different in NPC compared with gastric carcinoma.

In our EBV-positive carcinoma cases, the seroprevalence rate of VCA-IgA was higher in the patients with tumors in the cardia than in the patients with gastric tumors at other sites. Furthermore, the seroprevalence rates and GMTs of VCA-IgA in the EBV-positive carcinoma cases were significantly higher than in the EBV-negative carcinoma cases, while the seroprevalence rate of EA-IgA was very low in both the EBV-positive and -negative cases. We cannot deny the possibility that this phenomenon was caused by chance, since we examined various combinations of antibodies and tumor sites without any a priori hypotheses. The seroprevalence rates and GMTs of VCA-IgA in the EBV-negative carcinoma cases showed no difference according to tumor site.

A drawback of our study is that the blood samples of the EBV-negative patients with carcinoma were collected within 2 weeks after gastrectomy, while blood samples in the EBV-positive patients with carcinoma were taken before surgery. It is unlikely, however, that the methodological difference affected the comparison of antibody distributions between the EBV-positive and -negative carcinoma patients because it is unthinkable that EBV-specific antibody titers in the EBV-negative patients rapidly declined within 2 weeks, even if gastrectomy affects the magnitude of the antibodies. This conclusion is also supported by the results of the follow-up study of the seven EBV-positive patients with carcinoma, showing no significant change in the EBV-specific antibody titers during the period between cancer diagnosis and 1 month after gastrectomy. Tamada et al. [1984] observed decreased EBV-specific antibody titers in treated NPC patients, though not until 6 months after treatment.

In summary, the present study shows that EBV reactivation occurs in EBV-positive and -negative patients with carcinoma. The magnitude of the reactivation, however, is larger in the former than in the latter cases. Furthermore, since the high seroprevalence rate and GMT of VCA-IgA were noted only in the EBV-positive patients with carcinoma, it appears that VCA-IgA can act as a specific marker of immune response to EBV in EBV-positive patients with gastric carcinoma. Thus, our findings support the hypothesis that if EBV is involved in the development of EBV-positive gastric carcinoma, the EBV reactivation occurs in vivo. Further investigation is needed to clarify the etiological roles of EBV-positive carcinoma.

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